ON NITRIFICATION[1]

III. THE ISOLATION AND DESCRIPTION OF THE NITRITE FERMENT[2]

AUGUSTO BONAZZI

(WITH PLATE XIV)

Introduction

As a part of the physiological investigations upon nitrification carried on in this laboratory, the isolation of pure cultures of the nitrite-forming organisms became a necessity. Since difficulties in the isolation of these organisms have been encountered by many workers in the field of soil bacteriology, and since no accurate description of the organisms of nitrification from North American soils has been published as yet, it was deemed advisable to describe the organisms responsible for the formation of nitrites in the Ohio soils and the methods used in their isolation.

The following contribution, which is the result of a long period of study, is here given to describe an organism, capable of forming nitrites from ammonia, isolated in a pure state from Wooster soil after many unsatisfactory attempts.

Historical

In 1890 JORDAN and RICHARDS (4) in Massachusetts stated that they had isolated a nitrifying organism which was capable of oxidizing ammonia salts to nitrates when grown in solutions free of organic matter. The dilution method was the one relied upon by these investigators for obtaining pure cultures. Their description of this nitrifier is here reproduced.

[1] Contribution from the Laboratory of Soil Biology, Ohio Agricultural Experiment Station.

[2] The first paper of this series dealing with the subject of nitrification appeared under the title "Preliminary observations" in Ohio Agric. Exper. Sta. Technical Bull. 7. 1915. The second paper under the title "Intensive nitrite formation in solution" appeared in Jour. Bacteriology 1918. Thanks are due Dr. E. R. ALLEN, in whose laboratory this work was undertaken, for kind advice and criticism.

The bacilli are short, of a slightly oval shape, and vary from 1.1 μ to 1.7 μ in length; they are about 0.8–0.9 μ broad. They are grouped characteristically in irregular clumps and are held together by a jelly-like material. Each aggregation is indeed a typical zooglea. The aggregations of bacteria were found chiefly in the bottom of the flasks, as was also the case with the organism described by WINOGRADSKY. . . . . On one important point there appears to be a difference between our results and those reached by the above mentioned investigators. The organism discovered by them oxidizes ammonia to nitrite, but carries it no further. Our flasks give complete oxidation to nitrate. . . . . We are not even prepared to say that there may not have been a mixture of two or more species in our flasks, all agreeing closely in morphological characters, and in giving no growth on gelatin, but differing in important physiological respects.

This statement makes it quite clear that probably their cultures were a mixture of the two forms isolated and separated by WINOGRADSKY (11). No lengthy description of the organisms isolated by the Russian investigator is here necessary, and a summary review of his findings (12) will suffice. The nitrite-forming organisms from different parts of the world were divided by WINOGRADSKY in two genera and several species as follows: *Nitrosomonas* (*N. europeae* and *N. javaniensis*), from the Orient; and *Nitrosococcus*, from the Occident. *Nitrosomonas* received by far the greatest attention from WINOGRADSKY, while *Nitrosococcus*, which is most important to American economy, he only incompletely described. The soils from which WINOGRADSKY isolated his *Nitrosococcus* were from Campinas (Brazil), Quito (Ecuador), and Melbourne (Australia).

Samples of North American soils were not studied, and it is surprising that no description of organisms from such soils has been attempted since the time of the discovery of the active agents of nitrification. It is true that JORDAN and RICHARDS gave a description of the sewage organisms they were working with, but their description does not conform to the one given by WINOGRADSKY of the South American organisms. It is evident, therefore, that, were it only from a geobotanical standpoint, the description of the organisms from the Northern Hemisphere presents some importance.[3]

[3] The investigations reported by OWEN, WM. L. (The effect of carbonates upon nitrification. Georgia Exper. Sta. Bull. 81. 1–42. 1908), should not be overlooked in this connection. Unfortunately it must be admitted that the photographic reproductions of the organisms which he found in his flasks remind us only of a mixture of

The organism from Quito soils had, according to its discoverer, the following characters: a large coccus 1.5–1.7 μ in diameter, appearing larger in the living state than in the stained preparations. According to WINOGRADSKY this was probably due to a thick gelatinous membrane which did not stain or become invisible on desiccation. Typical zooglea, as those of *Nitrosomonas*, were never found, and the motile stage was never observed in liquid cultures of this organism. Corresponding to this behavior in solution, the colonies on silica jelly were only of one form, and were never seen to simulate the "clair colonies" of the *Nitrosomonas*. Surface colonies had the appearance of a yellowish liquid, and like the deep colonies were also made up of free cells. The organisms from Campinas and Melbourne showed the same characters, but were only different in size from the Quito organism, the Campinas coccus attaining a size of 2 μ in diameter, the Melbourne organism not quite 1.5 μ in diameter (a little smaller than the other two, WINOGRADSKY **12, 13**).

From this cursory review of the descriptions of the organisms it is evident that little is known, especially with regard to their cultural characters. A footnote in WINOGRADSKY'S paper (**12**) describes a peculiar linking of the cells of the Campinas organism which the author only observed once, and was not therefore in a position to study carefully. It should be mentioned also that this same Campinas organism gave a "trouble sans motilité" which WINOGRADSKY could not explain.

Criterion of purity

One difficulty encountered in the present work was the establishment of a criterion of purity, a standard sufficiently accurate to allow its general acceptance. The words "pure culture" in bacteriological literature are used to indicate such a culture as is made up completely or almost entirely of cells of the same type,

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various ubiquitous bacteria, which were not active in nitrification. The actual nitrite and nitrate production in the cultures studied by OWEN was so slight, to justify their being considered non-nitrifying cultures, that this fact, together with the heterogeneous bacterial contents, points to the possibility that he might not have possessed pure cultures of nitrifying organisms.

showing within the limits of individual variations in the same species the same physiological and morphological characters. Although theoretically a "pure culture" is that culture originated monocytogenetically, in practice it is seldom obtained. True pure cultures are only possible when the development of one single cell into a colony is controlled directly by microscopical observation; in ordinary technic this is not done. Although these requisites are necessarily of the same importance when "pure cultures" of nitrite- and nitrate-producers are desired, the term "pure culture" of nitrifying bacteria does not convey this same criterion of "absolute purity."

The simple fact that WINOGRADSKY found the nitrate- and nitrite-formers of different lands to possess in common the character of not growing in bouillon, in spite of their morphological differences, and that he made of this a criterion of purity, is enough to show the misleading interpretation given to the term "pure culture." The criterion of purity formulated by WINOGRADSKY (**12**) is as follows: "Introduce a loopfull of a nitrified culture in ordinary bouillon and keep at 30° C. during 10 days; at the end of this time the bouillon must not show turbidity. The purity of the nitrifying organisms is then proven."

That this criterion of purity is rather indefinite is shown by the following statement from JORDAN and RICHARDS, who, discussing the purity of some of their cultures, state that "we are not even prepared to say that there may not have been a mixture of two or more species in our flasks, all agreeing closely in morphological characters, and in giving no growth on gelatin, but differing in important physiological respects." OMELIANSKI (**6**) states that "after renewed ammonia additions it is necessary to control the purity by microscopical examinations as well as reinoculation in bouillon."

Since WIMMER (**9**) found a culture of nitrifying organisms to prove pure on bouillon of one reaction, while this same culture proved impure when tested on bouillon of a more alkaline reaction, the author has adopted in his work with nitrite-formers the following cardinal points which form the basis for his criterion of the purity of a culture: (1) the culture must be in full nitrification

before any attempt to determine the purity by the bouillon method is made; (2) the bouillon used in testing for purity should be of an alkaline reaction; better results would be obtained if the testing were made in bouillon of different reactions; (3) the time limit necessary for absolute reliability for the growth in bouillon should be fixed at 10 or more days; (4) the inoculum used in testing for purity by the bouillon method should be as large as permissible by ordinary technic; (5) microscopical examination must reveal a picture uniform within the limits of individual morphological variation in the species and within the different phases of the same organisms, provided such phases are established as correct.

A study of the growth of different organisms on the same medium and the description thereof is often relied upon as a diagnostic method. At best this method is unsatisfactory and of limited application. This is plainly shown by the fact that we cannot attempt to compare, on the same basis, the ordinary saprophytic and parasitic organisms with such organisms as will not grow on the media used in ordinary laboratory practice. The known organisms of the latter type are not numerous at present, but doubtless they are quite abundant in nature. The nitrite-formers of WINOGRADSKY are to be classified among the latter, together with the not less peculiar *B. oligocarbophilus*, *B. pantothropus*, and some organisms of the oligonitrophilus, sulphur, and iron groups. In the case of these organisms a study of their morphology in a few special media will lead to more accurate and reliable results than any attempt made to grow them in bouillon, gelatin, or agar of the ordinary composition.

Having presented the description of the South American nitrite-formers, and the criteria of purity followed in the present work, a description of the organism isolated to comply with these rules, from a North American soil, will be given after a discussion of the isolation technic.

Experimental

After establishing nitrification in solution by means of a small soil inoculum, the growth of the organism concerned was stimulated by continued additions of new doses of ammonium sulphate, new

ammoniacal salt being added only if the culture showed no ammonia reaction when tested with Nessler's reagent. From these enrichment cultures new cultures were started in solution with small inoculi. The culture solution used in all this work was the one recommended by OMELIANSKI (6), of the following composition: H_2O, 1000 cc.; $FeSO_4$, 0.4 gm.; $MgSO_4$, 0.5 gm.; K_2HPO_4, 1.0 gm.; NaCl, 2.0 gm.; $(NH_4)_2SO_4$, 2.0 gm.

Filtration of the solution was deemed unnecessary, since the addition of $MgCO_3$ milk after sterilization would make the practice useless. Growth was not impaired by preliminary filtration of the medium. After several transfers in solution, attempts were made at the isolation of pure cultures by plating in ammonium sulphate washed agar. Two consecutive platings in this medium

TABLE I

Soil	0	Silica jelly plates	63
Solution	19	Solution	66
Solution	24	Silica jelly plates	71
Washed agar plates	34	Solution	76
Washed agar slants	40	Solution	84
Washed agar plates	50	Solution	86
Solution	59		

gave cultures which did not fulfil the requirements of the "criteria of purity." It was only after 3 consecutive platings in a silicic acid jelly medium (6) that cultures were obtained which proved pure to the criteria. The genealogic succession of the cultures is shown in table I, where the series number accompanies the description of the medium used in each generation studied.

It was only a culture of series 86 in solution that, when inoculated in bouillon of a +1 per cent and a −1 per cent reaction, gave no growth whatsoever, either before or after the 10-day period had elapsed. Microscopically no foreign forms were seen, and a perfectly pure culture was indicated by this test. Unfortunately, after this culture was obtained it was observed that its action was relatively slow, and in subsequent transfers the inoculi used were very large, 1 or 2 cc. Notwithstanding these precautions, after the cultures had been transferred once or twice again they

gave no more the characteristic nitrite formation. The genealogic succession is represented in table II.

Tests made in bouillon (of a + and − reaction) proved cultures of series 90 and series 94 to be absolutely pure. Cultures of series 86 were inoculated with 1 cc. of the mother culture, those of series 88 with 1 cc., those of series 90 with 1.5 cc., those of series 94 with 2.5 cc., and those of series 101 with one large loop of such a shape that it contained 2–3 drops of solution. The reaction of cultures from series 94 was questionable, and microscopic examination of some cultures of this series showed the organism to be very slow growing, so slow in fact as to allow a contamination to enter during the manipulations subsequent to the first test for purity. The nitrite formation of culture 101 was nil after nearly a month, and its cellular contents nil. That the loop used in this

TABLE II

Solution	86	Solution	94
Solution	88	Solution	101
Solution	90		

case was large enough for a successful inoculation, provided the mother cultures were growing well, is proved by the thousand other successful inoculations by this method.

The characters of the organisms isolated during the preceding series of generations are described later. Although these organisms in the pure state tended to lose their nitrifying power when cultivated in solutions at rest, by cultivating them in ignited soil, to which the ordinary OMELIANSKI solution and magnesium carbonate were added, it was possible to stimulate their action considerably. The cultivation of these same organisms in solutions undergoing a slow rotary movement and constant aëration proved them to possess a very strong nitrifying power. Organisms which were slowly losing their power of nitrification in the ordinary laboratory condition, as they approached a state of purity, were soon made to increase this power, up to an intensive nitrification, by appropriate means (BONAZZI 2).

Cultures in solution

Macroscopic examination of young solution cultures reveals no indication of bacterial growth, and it is not until much ammonia has been oxidized to nitrite that any macroscopic growth is apparent. By observing the bottom of the culture flask 30–40 days' old an inconspicuous slimy deposit is visible which is easily dispersed by shaking. Before this point is reached no clouding or movement of the solution is visible, such as the "trouble" imparted to the solution by the "monad stage" of the European organisms. In fact no distinction can be drawn between the inoculated and non-inoculated flasks incubated for the same period of time. This similarity is maintained throughout the life of cultures which have been kept in this laboratory for over 2 months. No surface growth is visible, even when the cultures have attained very old age. Two cultures, which had nitrified 74.41 and 48.10 mg. of ammoniacal nitrogen while at rest, showed no surface growth whatever. No motility can be observed in the cultures, and this is in conformity with the behavior of the South American cultures described by WINOGRADSKY. In all the work with solutions at rest we have adopted the use of 20 or 25 cc. of solution in 250 or 300 cc. Erlenmeyer flasks, since this depth of solution furnishes a relatively good aëration.

Cultures on solid media

Several solid media have been tried in this laboratory in the hope that a satisfactory method could be found which would be advantageous to the speedy growth of the organism of "nitrosofermentation." Among others there were tried the paper pad method and the gypsum block method of OMELIANSKI (6), the magnesium carbonate block method of PEROTTI (7), the magnesium carbonate and ammonium-magnesium-phosphate block method of MAKRINOFF (5), the ammonium sulphate washed agar method of BEIJERINCK (1),[4] the silicic acid jelly method of STEVENS and

[4] The method of BEIJERINCK was also modified so that the washed agar only came in contact with the salts a few minutes before inoculation. This was accomplished by incorporating, just before plating, the necessary quantity of salts dissolved in 5 cc. of water with 5 cc. of a melted washed agar jelly, mixing thoroughly, inoculating, and plating. The action of the salts on the agar at high temperatures was thereby avoided.

TEMPLE (8), and the silicic acid jelly method of WINOGRADSKY (10). By far the best and most reliable results were obtained with WINOGRADSKY'S silicic acid method, which has now been adopted in this laboratory as the best among all those tried. When an impure enriched culture is inoculated in silicic acid jelly plates superficially, very little visible growth takes place. The point of inoculation only assumes a perlaceous aspect and does not change with age.

When the inoculum is incorporated with the nutritive jelly and incubated at 30° C., no growth is visible before 11 or 12 days have elapsed from the time of inoculation. It is only after this period that small colonies are visible to a magnification of 75 diameters, barely distinguishable in the thick mass of crystals formed in the plate. After the lapse of a few days more the colonies reach a size of $\pm 224 \times 160\,\mu$; when observed by transmitted light they have the appearance of small yellowish masses surrounded by a colorless halo, which is due to the solution of the $MgCO_3$. This characteristic may be utilized as a means of differentiation in the identification of the colonies (fig. 1). The colonies have at first diffused outlines, and slowly take on a more definite form, their appearance being well shown in figs. 2 and 3. They never assume a hard consistency, remaining always soft. Colonies of 1 mm. diameter have been obtained by renewing the $(NH_4)_2SO_4$ in the plates when necessary, by the method recommended by OMELIANSKI.

Hanging drop cultures

Hanging drop cultures show no motility, even when the material is taken from mother cultures which are undergoing at the time a strong nitrification (figs. 7 and 8). Material prepared for microscopical observation from cultures undergoing intensive nitrification on a klinostat (BONAZZI 2) only showed a slow vibration of the cells, easily ascribable to Brownian movement.

Microscopical aspect of organism

In the beginning of the investigation the solution cultures were searched for any growth which might resemble the typical zooglea or the typical monad forms described by WINOGRADSKY

for the organisms of the Old World. Failing in the search, attempts were made to find any organism which, by its constant abundance in the cultures, might prove to be the principal type. On account of the slow growth of the organisms of nitrosofermentation in the ordinary laboratory conditions, it was not until nearly pure cultures were obtained that I observed, mixed with and imbedded in the salt deposit, a large coccus form, much resembling the one described by WINOGRADSKY as peculiar to South American soils. The strong "ferment power" of this organism had aided it in escaping attention in impure cultures. These megalococci are shown in fig. 6. They are large, $\pm 1.25\,\mu$ in diameter, and of a slightly irregular roundish form. Some are occasionally found which appear to have a triangular section, but closer observation reveals them to be clumps of smaller cells arranged so as to simulate a tetrahedron.

The microscopical examination of material from cultures in full nitrification, answering to the criterion of purity, is the most instructive. Fig. 5 shows the appearance of the megalococci at this time; they are composed of a thick gelatinous mass in which are imbedded small granules. Very often seemingly bipolated bodies are imbedded in this jelly, but on closer examination these polar bodies appear to be, not small cocci, but diplococci, the true living active units (figs. 4, 5). Material from intensive cultures, observed fresh in strong Meissner solution, showed these structures very plainly stained differentially and larger than in the dried preparations, since on drying the gelatinous mass seems to lose its thick structure. This gelatinous coat has a thickness that equals the diameter of the cells imbedded in it, and it takes on a bluish tinge in iodine, while the imbedded cells stain golden yellow in the same reagent.

When the cultures are in full and strong nitrification, the megalococci give rise to the small cocci which we will name β. The latter are clearly shown in fig. 5. These small β forms, which were at first imbedded in a thick gelatinous mass, forming the large α cocci, are set free and begin independent life, leaving the empty sheaths which are occasionally to be seen in stained preparations. Some of the β forms have been observed to take up a

gelatinous coating and revert to megalococci. This cycle recalls a little the cycle followed by the organisms of Java soils, as related by WINOGRADSKY (**12**, figs. 10, 12).

The staining methods adopted in this study are similar to the methods recommended by WINOGRADSKY. Although several of the ordinary staining solutions were tried, none seemed to give as good and clear pictures as the malachite-green and gentian-violet method. The salts, which are generally placed on the slide with the bacterial preparations, especially when solutions are the origin of the material studied, are not stained by this method. The technic is as follows: The coverglass preparation, flame fixed, is mordanted for 1 minute in the cold with a 0.25 per cent solution of malachite-green in distilled water, washed with cold water, and stained cold with a 0.25 per cent water solution of gentian-violet for 1 more minute. Washing is then done rapidly with water previously heated to 50–60° C. This washing takes out any coloration of the salts which might cloud the microscopic field. Preparations are thus obtained which stain the jelly of the megalococci a deep purple and the small cocci of the β type a purple-black color. Treatment with acid is not necessary to dissolve the salt formations. In the fresh unstained state the cells are not easily visible, and a search for them often proves unsuccessful. The color differentiation mentioned, obtained in Meissner solution, constitutes what seems to be the best and most reliable one for a study of the organisms in hanging drop preparations.

Temperature relations

The thermal death point of the organism studied was found to lie between 50 and 55° C., when the vitality of the organism, after heating 5.5 minutes at the required temperature, was tested at rest in OMELIANSKI's solution containing basic magnesium carbonate. An additional study of the resistance of the organism to heat was also made. One cc. portions of a strong nitrifying culture were placed in sterile vials, heated at given temperature for given lengths of time, and then quickly cooled in stone cold water, the depth of the solution layer being 4–5 mm. over a surface of ± 2.8 cm^2. Heating was done in ovens, and tests for

the vitality of the cultures were made by inoculating all the heated material in large test-tubes containing 25 gm. of sterile coarse ignited soil and $MgCO_3$ kept in a slanting position by 10 cc. of sterile OMELIANSKI solution. Duplicates were run for each treatment. When the cultures were tested for the formation of nitrite after 12 days of incubation at 30° C., the results given in table III were obtained.

Although the duration of incubation was not very long, the nature of the medium was such as to allow complete nitrification in all cases where full inoculum was used. The fact that a heating of 40 minutes at 50° C., a heating of 10 minutes at 55° C.,

TABLE III*

Temperature of heating	Time of heating in minutes					
	2	5	10	20	40	60
45° C..............	+	+	+	+	+	+
50° C..............	+	+	+	+	o	
55° C..............	+	+	?			
62–65° C...........	+	o				

*The + indicates a strong nitrite formation, ? a questionable nitrite formation, and o no nitrite formation.

and a heating of 5 minutes at 62–65° C. gave questionable or negative nitrification, while all other cultures gave complete nitrification, is very significant.

BOULLANGER and MASSOL (3) found the thermal death point of the organism of nitrosofermentation isolated in their work to lie between 45° and 50° C. when the heating continued for 5 minutes. It is seen, therefore, that the organism isolated from Wooster soils resists higher temperatures and longer periods of heating than the one which constituted the object of their studies. The difference in apparatus used might lead to slight variations in the T.D.P. determinations.

The incubation of all the cultures of nitrite-formers cultivated in this laboratory has been done at 28–30° C. At this temperature cultures were obtained which nitrified as much as 8.04 mg. of ammoniacal nitrogen in 26 days of incubation at rest.

Discussion

The results of this study on the morphology of the organisms of nitrosofermentation, active in the North American soils, are not easily reconciled with the results obtained 28 years ago by JORDAN and RICHARDS. The discrepancy between the results is undoubtedly to be ascribed to two reasons: (1) incomplete description of the organisms isolated from sewage in Massachusetts, and (2) the probable presence in those cultures of organisms capable of transforming nitrites to nitrates. The possibility of a mixed culture was admitted by JORDAN and RICHARDS on the basis of the absence of nitrite formation during nitrification.

The resemblance of the megalococcus isolated by WINOGRADSKY from the South American soils to the megalococcus for the North American soils is quite striking. Excepting the slight difference in size existing between the two forms, all other characters are common to both. The variation in the life cycle of the two organisms is probably only apparent, since the true cocci, which have been named β by the author, might have escaped WINOGRADSKY'S attention. It is to be regretted that the Russian investigator was not able to study the nitroso-organisms from the New World as thoroughly as he did those from the Old World, and that he was obliged to publish only an abridged description of the former types. In the present work an attempt was made at the completion of his work, and results have been reached which corroborate the postulations to be made from his findings, in such a way filling a gap in our knowledge of the distribution of the nitrite-forming organisms. That the organism isolated in Wooster can be classed as a species of the genus *Nitrosococcus* seems to be justified by these findings. The comparative study of the species of the genus *Nitrosococcus* will have to be deferred to a time when a careful study will be possible of the organisms of nitrosofermentation in South America and Australia.

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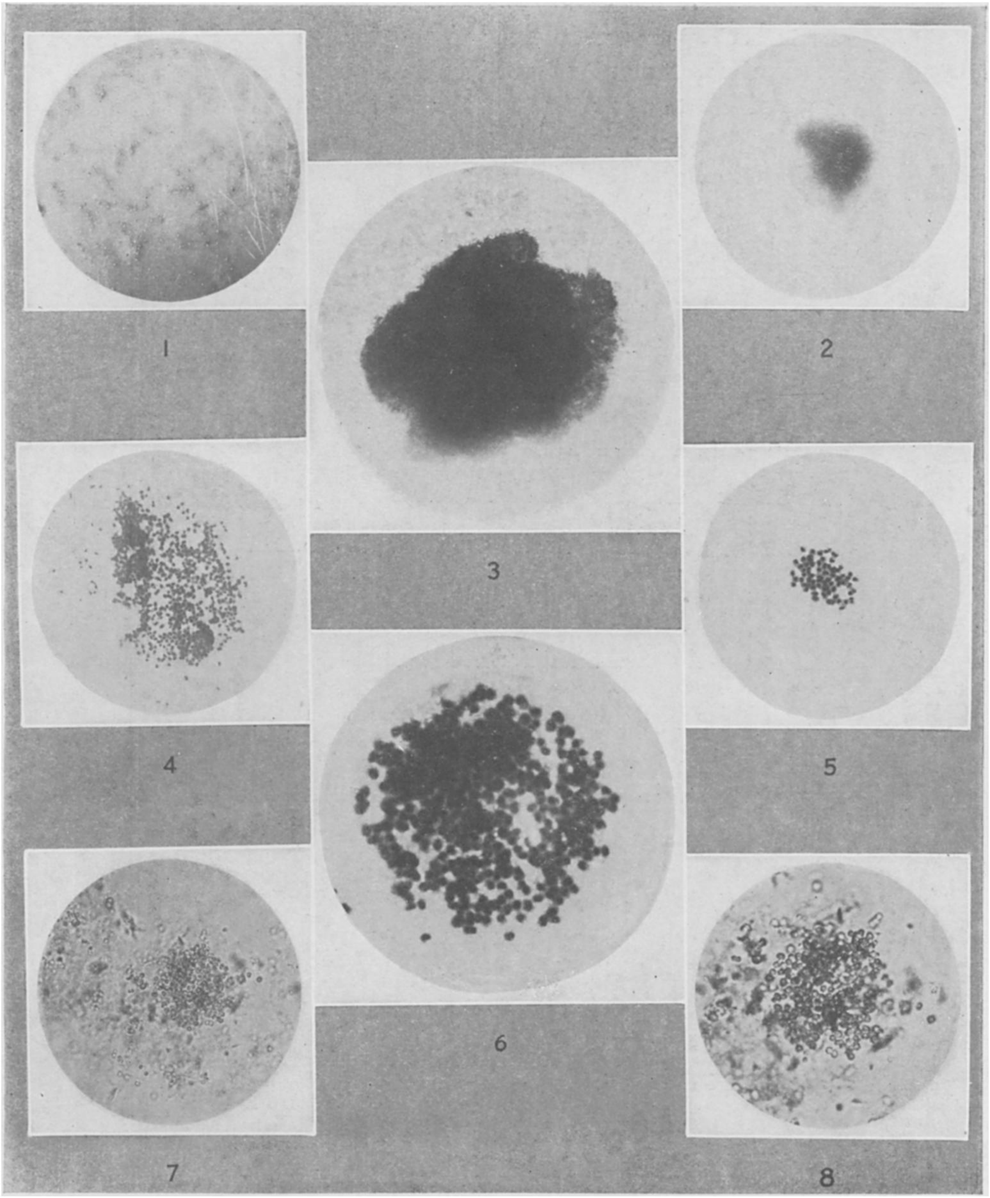

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EXPLANATION OF PLATE XIV

The photomicrographs were made with the kind assistance of Mr. VICTOR DYE, photographer of the Station.

FIG. 1.—Portion of silica jelly plate, showing colonies of *Nitrosococcus* after several additions of ammonium sulphate; solubilization of magnesium carbonate in vicinity of colonies very evident; ×1.5.

FIG. 2.—Colony of *Nitrosococcus* on silica jelly plate, showing loosely granular structure, as seen against a source of light; ×77.

FIG. 3.—Another colony on silica jelly; ×175.

FIG. 4.—*Nitrosococcus* from culture undergoing intensive nitrification, showing lack of zooglea formation; stained by malachite-green–gentian-violet method of WINOGRADSKY; ×820.

FIG. 5.—*Nitrosococcus* from culture undergoing intensive nitrification, showing small β forms imbedded in thick gelatinous membrane; cocci and diplococci; stained by malachite-green–gentian-violet method of WINOGRADSKY; ×820.

FIG. 6.—*Nitrosococcus* from culture undergoing intensive nitrification, showing typical megalococci; in some cases the small forms are seen appearing in thick gelatinous membrane, stained by malachite-green–gentian-violet method of WINOGRADSKY; ×1600.

FIG. 7.—*Nitrosococcus* in hanging drop preparation, stained with Meissner's solution; ×740.

FIG. 8.—*Nitrosococcus*, same as no. 7; ×1200.